

CD4⁺CD25⁺CD62L⁺CD45RO⁻ T Cells From Thymus, Cord Blood and Peripheral Blood as Precursor of CD4⁺CD25^{High} Regulatory T Cells

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ABSTRACT

In the present study, we have investigated the possible mechanisms of origin, continuous generation and maintenance of naturally occurring CD4⁺CD25^{High} regulatory T cells in the periphery. The present study has investigated the presence of cell precursors for regulatory T cells in thymus, cord blood and peripheral blood. We have identified in the three anatomic compartments the presence of a cell population defined by a CD4⁺CD25⁺CD62L⁺CD45RO⁻ phenotype. This population represented 70-80% of the CD4⁺CD25⁺ T cells in cord blood and 20% in peripheral blood and in the thymus. In cord blood these cells also expressed CTLA-4 (27%±4.75, n=6). More importantly, high levels of expression of FoxP3 were consistently observed in this cell population isolated from cord, peripheral blood and thymus. These results strongly suggested that these cells were regulatory T cells. We then assessed the proliferative capacity and the suppressive function of sorted purified CD4⁺CD25⁺CD62L⁺CD45RO⁻ cells from cord blood and peripheral blood. We observed that these cells retained proliferative ability and had no suppressive function. In order to support the hypothesis that this cell population may serve as a precursor of Treg, we developed an *in vitro* culture system to induce the maturation/differentiation of sorted purified CD4⁺CD25⁺CD62L⁺CD45RO⁻ using soluble anti-CD3/anti-CD28 stimulation in the presence of IL-2. Interestingly, after one week of culture, the cells had acquired the phenotype of typical Treg (CD4⁺CD25⁺CD45RO⁺) and more importantly, they had also acquired the capacity to suppress the proliferation of CD4⁺CD25⁺ cells following polyclonal stimulation or in mixed lymphocyte reaction (60%±3.9 suppression of proliferation mediated by cell precursors from the cord blood, n=7; 87.5%±3.8 suppression mediated by precursors cells isolated from peripheral blood, n=2). The levels of suppression of proliferation mediated by these cell population matured *in vitro* were in the same range of the suppression mediated by freshly isolated mature Treg (88%±5.2, n=6). Therefore the existence of a peripheral precursor of the T reg cell population with full proliferative capacity likely represents the *in vivo* mechanism leading to continuous generation of mature T reg cells and the maintenance of a stable peripheral pool. The identification of a precursor T reg cell population with proliferative capacity provides an ideal potential target in order to develop intervention strategies that may substantially influence the number of mature suppressive T reg cells and thus their immunoregulatory role.

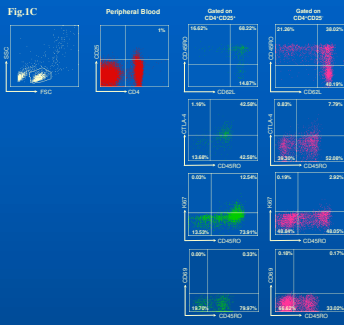
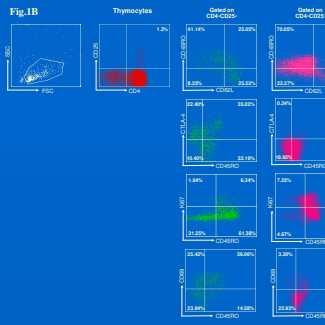
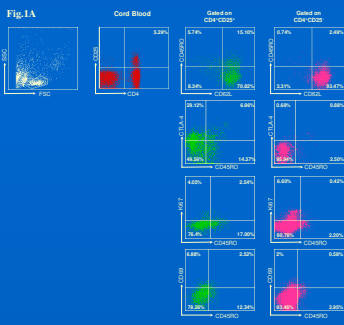
Introduction

The mechanisms responsible for the generation and maintenance of a stable pool of natural occurring CD4⁺CD25⁺ regulatory T cells in the periphery are still unclear. Here we report the phenotypical and functional characterization of a cell population that may serve as precursor of human CD4⁺CD25⁺ regulatory T cells in thymus, cord blood and peripheral blood featured by the CD62L⁺CD45RO⁻ phenotype and we have also defined the requirements for their maturation.

Results:

Phenotypic and molecular characterization of T reg cells:

We have performed initially phenotypic and molecular characterization of T reg cells in cord blood. Consistently with previous studies we have found that 3-5% of cord blood CD4⁺ T cells expressed CD25 and were mostly CD45RO⁻ and about 20% were CD45RO⁺ positive. In addition about 90% of these cells were CD62L⁺ positive and about 40% of CD25⁺CD45RO⁻ and 30% of CD25⁺CD45RO⁺ cells expressed CTLA-4 (referred to Fig.1A). The cell population gated was typical of small lymphocytes and the expression of markers of cell cycle as Ki67 and of recent activation as CD69 was almost absent in CD4⁺CD25⁺ cells. We then investigated the presence of CD4⁺CD25⁺CD62L⁺CD45RO⁻ cell population in the thymus and in the peripheral blood. Phenotypic analysis showed that more than 60% of CD4⁺CD25⁺CD45RO⁻ cells from thymus expressed CTLA-4. There was no evidence of cycling activity (Ki67) within CD4⁺CD25⁺CD45RO⁻ respect to the CD4⁺CD25⁺ cells. However nearly 50% of CD4⁺CD25⁺CD45RO⁻ expressed the CD69 recent activation marker (Fig.1B). In the peripheral blood about 85% of CD4⁺CD25⁺ were CD62L⁺ positive. Nearly 80% of CD4⁺CD25⁺ were CD45RO⁻ and of interest 20% were consistently found CD45RO⁺ positive. Furthermore CD4⁺CD25⁺CD45RO⁻ did not express significant amount of markers of cell activation such as Ki67 and CD69 (Fig.1C). **From all the three compartments we had evidence of the existence of a new population characterized by the CD4⁺CD25⁺CD62L⁺CD45RO⁻ phenotype coexisting with the well known CD4⁺CD25⁺CD62L⁺CD45RO⁺ regulatory T cells.**



FoxP3 expression in different CD4⁺ populations:

Then we wanted to investigate whether the two populations of CD4⁺CD25⁺ found in the 3 different anatomic compartment were Treg cells. In order to address this issue sorted purified CD4⁺CD25⁺CD62L⁺CD45RO⁻ and CD4⁺CD25⁺CD62L⁺CD45RO⁺ cell populations from cord blood (Fig.2A), CD4⁺CD25⁺CD62L⁺CD45RO⁻ and CD4⁺CD25⁺CD62L⁺CD45RO⁺ cell populations from peripheral blood (Fig. 2B) and CD8⁺CD4⁺CD25⁺CD45RO⁻, CD8⁺CD4⁺CD25⁺CD45RO⁺ and CD4⁺CD25⁺CD62L⁺CD45RO⁺ cell populations from thymus (Fig.2C) were assessed by quantitative real time PCR for the expression of FoxP3 mRNA that is selectively present in Treg cells in resting conditions. High levels of FoxP3 expression were only found in the CD4⁺CD25⁺ cell populations, one characterized by the typical phenotype of naturally occurring T reg cells (CD45RO⁻) and the other by the CD45RO⁺, which showed as well the lineage molecular marker of CD4⁺ Treg and might represent a precursor of the mature counter-part.

Fig.2A FoxP3 mRNA expression in sorted purified subpopulation from Cord Blood

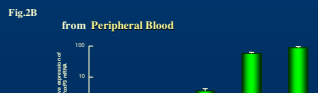
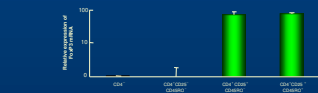
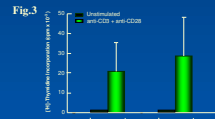


Fig.2C from Thymus

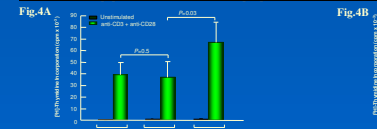


Functional characterization of cord blood and peripheral blood CD4⁺CD25⁺CD62L⁺CD45RO⁻ cells:

We investigated the **proliferative capacity** and **suppressor function** of this population. For this purpose cord blood cells were sorted purified in CD4⁺CD25⁺CD62L⁺CD45RO⁻ and CD4⁺CD25⁺CD62L⁺CD45RO⁺ cells and the proliferative capacity was assessed following stimulation with anti-CD3 plus anti-CD28 antibodies (1µg/ml each) in presence of irradiated syngeneic cord blood mononuclear cells for 3 days, adding [³H]-Thymidine for the last 16 hours. Results as mean ± SE of 4 independent experiments are shown in Fig. 3. **The proliferative capacity of the two populations has not been found significantly different after anti-CD3 plus anti-CD28 stimulation.**

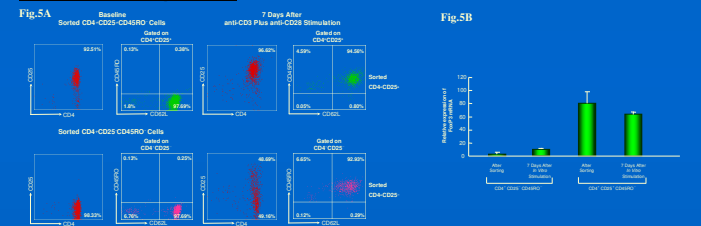


We then investigated the **suppressor activity** of the CD4⁺CD25⁺CD62L⁺CD45RO⁻ T reg cells from cord blood and peripheral blood. Sorted purified CD4⁺CD25⁺CD62L⁺CD45RO⁻ cells from cord blood or from peripheral blood were co-cultured with allogeneic blood mononuclear cells or with syngeneic CD4⁺ T cells and irradiated PBMC, respectively, in presence of anti-CD3 plus anti-CD28 antibodies (1µg/ml each). The levels of [³H]-Thymidine incorporation observed in the cultures containing stimulated blood allogeneic mononuclear cells alone or syngeneic CD4⁺ T cells with irradiated PBMC, in presence of the polyclonal stimulus for 3 days, were comparable to those observed in the co-cultures with the CD4⁺CD25⁺CD62L⁺CD45RO⁻. Results as mean ± SE of 7 and 3 independent experiments, respectively, are shown in Fig. 4A and 4B. **The CD45RO⁻ cell population from cord blood and peripheral blood CD4⁺CD25⁺ T cells did not show any suppressor activity.**



Induction of phenotypic and functional maturation of CD4⁺CD25⁺CD62L⁺CD45RO⁻ T cells:

We then investigated whether functional and phenotypic maturation could be promoted in the CD4⁺CD25⁺CD62L⁺CD45RO⁻ T cells. For this purpose, sorted purified CD4⁺CD25⁺CD62L⁺CD45RO⁻ and CD4⁺CD25⁺CD62L⁺CD45RO⁺ cell population from cord blood or peripheral blood were cultured under different stimulation conditions. Seven days following stimulation with anti-CD3 (1µg/ml) plus anti-CD28 (1µg/ml) in presence of exogenous IL-2 (20 U/ml) and irradiated PBMC, in presence of the polyclonal stimulus for 3 days, were comparable to those observed in the co-cultures with the CD4⁺CD25⁺CD62L⁺CD45RO⁺. Results as mean ± SE of 20 experiments is shown in Fig.5A). The expression of FoxP3 remained significant only in the CD4⁺CD25⁺ T cells also after maturation (Fig.5B). **Therefore, the CD4⁺CD25⁺CD62L⁺CD45RO⁻ T cells from cord blood and peripheral blood acquired a phenotype typical of mature Treg after anti-CD3 plus anti-CD28 *in vitro* stimulation in presence of IL-2.**

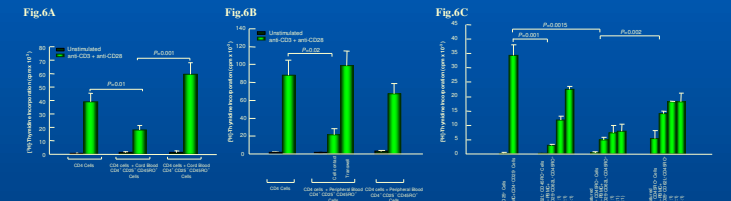


Test of suppression function of *in vitro* matured CD4⁺CD25⁺CD62L⁺CD45RO⁺ T cells:

The obvious step was to determine whether these cells had acquired suppressor function. The stimulated cell populations generated from sorted purified CD4⁺CD25⁺CD62L⁺CD45RO⁻ and CD4⁺CD25⁺CD62L⁺CD45RO⁺ from cord blood were co-cultured with allogeneic purified CD4⁺ T cells while CD4⁺CD25⁺CD62L⁺CD45RO⁻ and CD4⁺CD25⁺CD62L⁺CD45RO⁺ from peripheral blood were co-cultured with syngeneic purified CD4⁺ T cells both in presence of anti-CD3 (1µg/ml) plus anti-CD28 (1µg/ml) and irradiated peripheral mononuclear blood cells, 3 days later, cell proliferation in cultures was evaluated by [³H]-Thymidine uptake. A substantial suppression (about 60%) of the proliferation of allogeneic CD4⁺ T cells stimulated with anti-CD3 plus anti-CD28 antibodies was observed only in cultures containing the CD4⁺CD25⁺CD62L⁺CD45RO⁻ cells originated after *in vitro* stimulation of the CD4⁺CD25⁺CD62L⁺CD45RO⁻ T cells from cord blood. These results are representative of 7 independent experiments (Fig.6A). Similar results (70% ± 4 suppression) have been obtained using CD4⁺CD25⁺CD62L⁺CD45RO⁻ cells in cultures containing syngeneic CD4⁺ T cells (as well as in syngeneic CD4⁺CD25⁺ T cells) in presence of anti-CD3 plus anti-CD28 antibodies for 3 days. (Results from 3 independent experiments, Fig.6B).

We assessed the suppression function of the *in vitro* matured CD4⁺CD25⁺CD62L⁺CD45RO⁺ cells in cultures containing syngeneic CD4⁺ T cells following mixed lymphocyte reaction. Syngeneic CD4⁺CD25⁺ T cells were cultured with irradiated allogeneic peripheral mononuclear cells for 7 days and pulsed with [³H]-Thymidine for 16 hours (Fig.6C). Significant suppression function was observed only in presence of the CD4⁺CD25⁺CD62L⁺CD45RO⁺ population either fresh or *in vitro* matured (91%±0.7 and 85%±2.35, respectively).

Taken together these results suggest that the CD4⁺CD25⁺CD62L⁺CD45RO⁻ cells may serve as precursors for the naturally occurring suppressor T reg cells.



Conclusions:

- The major observations of this study include:
- the CD4⁺CD25⁺CD62L⁺CD45RO⁻ and CD45RO⁺ cell populations belonged unequivocally to the lineage of T reg cells.
 - the existence of a new lineage committed T reg cell population defined by the CD4⁺CD25⁺CD62L⁺CD45RO⁻ phenotype.
 - the TCR plus co-stimulatory molecules triggering resulted in the switch to the CD45RO⁻ phenotype and in the acquisition of suppressive function by the CD45RO⁻ T reg cells;
 - the presence of CD4⁺CD25⁺CD62L⁺CD45RO⁻ Foxp3⁺ cell population within the thymus demonstrated the existence of a cell population that may serve as precursor of Treg also in the thymus;
 - the existence of a putative precursor T reg cell population in the periphery that is neither anergic nor suppressive. The preservation of the proliferative capacity in the putative precursor may allow a continuous supply of mature suppressive T reg cells and may represent a mechanism of maintenance of a stable peripheral pool of T reg cells.
 - the identification of a non-anergic precursor Treg cell population provides an ideal potential cell target in order to develop intervention strategies that may substantially influence the number of mature suppressive T reg cells and thus their immunoregulatory role.

